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Certification of copy of documents relating to a patent application for industrial invention N. MI97A 002080

It is hereby declared that the enclosed copy corresponds to the documents as originally filed with the above mentioned patent application whose data are as from the enclosed filing certificate.

Rome, 04 November 1998

THE DIRECTOR OF THE DIVISION
Dr. Giorgio Romani
(signature)

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Serial No.: 09/508,277
Filed: March 7, 2000
Applicants: Danilo Porro et al.
Certified Copy of Italian Priority Application
Attorney Docket: 2027.547000/RFE

TO THE MINISTRY OF INDUSTRY, TRADE AND HANDICRAFT
CENTRAL PATENT OFFICE - ROME

Space to be covered with stamps
FORM A

APPLICATION FOR INDUSTRIAL INVENTION, FILING MISSING DOCUMENTS, ADVANCED AVAILABILITY TO THE PUBLIC

A. APPLICANT(S)
NAME BIOPOLO S.C. a.r.l. .. [SP]
SEATING IN Milano CODE 11442050156
2) NAME: [ ]
SEATING IN CODE

B. APPLICANT'S REPRESENTATIVE AT THE CENTRAL PATENT OFFICE
SURNAME, NAME:BIANCHETTI Giuseppe et al. FISCAL CODE:
NAME OF THE OFFICE: Studio Consulenza Brevettuale S.r.l.
STREET:Rossini NO. 8 TOWN: Milano ZIP CODE:20122
PROV.: MI

C. ADDRESSEE'S ELECTIVE DOMICILE:
STREET: N. TOWN: ZIP CODE: PROV:

D. TITLE proposed class(sect/cl/scl):[C07D] group/sub-group:[498/04]
"YEAST STRAINS FOR THE PRODUCTION OF LACTIC ACID"

ADVANCED ACCESS TO THE PUBLIC: YES[ ] NO[X]
IF REQUEST: DATE / / / RECORD N. [ ]

E. NAMED INVENTORS:

surname, name	surname, name
1) PORRO. Danilo	3) RANZI, Bianca Maria
2) BIANCHI, Michele	4) FRONTALI, Laura

F. PRIORITY

COUNTRY OR ORGANIZATION	KIND OF PRIORITY	APPL.N.	FILING DATE	ENCL Y/M
1)			/ /	
2)			/ /	

MISSING DOCUMENTS FILED:	FILING DATE	DOCUMENT N.
1)	/ /	
2)	/ /	

G. AUTHORIZED COLLECTING CENTER FOR MICROORGANISMS CULTURE

H. SPECIAL NOTES:

ENCLOSED DOCUMENTS:

	N. COPIES			
DOC. 1)	[1]	[PROV]	N.PAGES [27]	abstract and main drawing, description and claims (1 copy compulsory)
DOC. 2)	[00]	[PROV]	N.SHEET [06] OF DRAWING	drawing (1 copy compulsory if cited in the description)
DOC. 3)	[01]	[FOLLOWS]		power of attorney
DOC. 4)	[00]	[FOLLOWS]		designation of inventor
DOC. 5)	[00]	[FOLLOW]		priority documents with Italian translation
DOC. 6)	[00]	[FOLLOWS]		authorization or assignment act
DOC. 7)	[00]			full name of the applicant

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DATE	RECORD N.
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compare single priorities

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8. Payment receipts, Total amount Lira five hundred sixty-five thousand compulsory

FILLED IN ON 12.09.1997 **SIGNATURE OF THE APPLICANT(S)**

CONTINUES YES/NO [NO] SPADARO, Marco (signature)

CERTIFIED COPY OF THE PRESENT ACT IS REQUESTED YES/NO [YES]

PROVINCIAL OFFICE IND. TRADE HAND. OF MILAN CODE [15]

FILING CERTIFICATE: APPLICATION NUMBER MI97A 002080 Reg.A

The year NINETEEN-NINETY-SEVEN, this TWELTH day of the month of SEPTEMBER the above mentioned applicant(s) has(have) produced to me the undersigned the present application, consisting of N. [01] additional sheets for the granting of the overmentioned patent.

I. VARIOUS NOTES OF THE ATTESTING OFFICER

THE PETITIONER	**SEAL OF THE OFFICE**	**THE ATTESTING OFFICER**
(signature)	Seal of the Ministry of Industry, Commerce and Handicraft	A. Pagnoncelli (signature)

ADDITIONAL SHEET TO FORM A
ADDITIONAL SHEET N. [01] OF TOTAL SHEETS [01]
APPLICATION N. MI97A 002080 REG.A

A. APPLICANT(S) J.N.
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E. NAMED INVENTORS:

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F. PRIORITY

	COUNTRY OR ORGANIZATION	KIND OF PRIORITY	APPL.N.	FILING DATE	ENCL Y/M
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PETITIONER SIGNATURE: SPADARO, Marco
(signature)

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stry, Commerce and Handicraft

ABSTRACT OF THE INVENTION WITH MAIN DRAWING, DESCRIPTION AND CLAIMS

Application NO. MI97A 002080 Reg.B Filing date 12 September 1997

Patent N. Granting date / /

D. TITLE

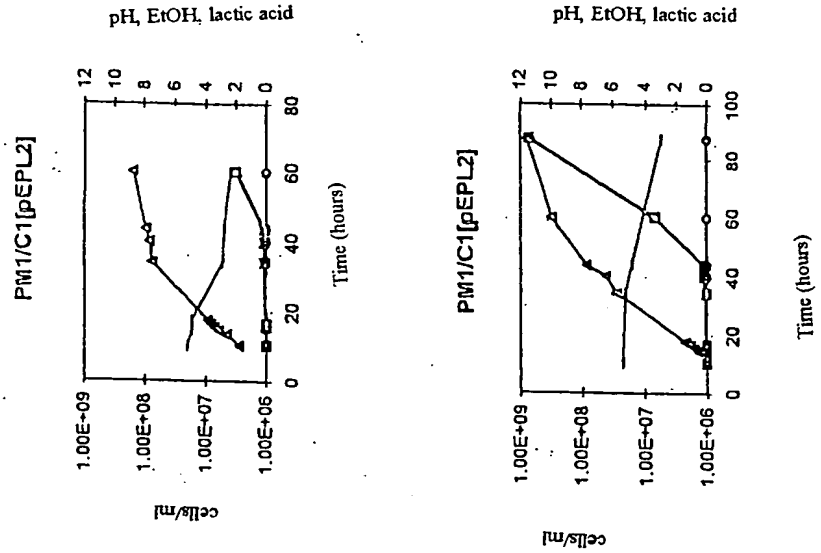
YEAST STRAINS FOR THE PRODUCTION OF LACTIC ACID

L.ABSTRACT

There is described a yeast strain lacking ethanol production activity or having a substantially reduced ethanol production activity, transformed with at least one copy of a gene coding for lactic dehydrogenase enzyme, functionally linked to promoter sequences which allow the expression of said gene in yeast.

DRAWINGS

Figure 5A & 5B



Description of the industrial invention having title:
YEAST STRAINS FOR THE PRODUCTION OF LACTIC ACID
in the name of BIOPOLO S.C. a.r.l.
seating in Milan

Background of the invention

The applications of lactic acid and its derivatives encompass many fields of industrial activities (i.e., chemistry, cosmetic, and pharmacy), as well as important aspects of food manufacture and use. Furthermore, today there is growing interest in the production of such an organic acid to be used directly for the synthesis of biodegradable polymer materials.

Lactic acid may be produced by chemical synthesis or by fermentation of carbohydrates using microorganisms. The latter method is now commercially preferred because microorganisms have been developed, that produce exclusively one isomer, as opposed to the racemic mixture generated by chemical synthesis. The most important industrial microorganisms, such as species of the genera Lactobacillus, Bacillus, and Rhizopus, produce L(+)-lactic acid. Production by fermentation of D(-)-lactic acid or mixtures of L(+)- and D(-)-lactic acid are also known.

During a typical lactic acid fermentation, there is an inhibitory effect caused by the lactic acid produced on the metabolic activities of the producing microorganism. Besides the presence of lactic acid, by lowering the pH value, also inhibits cell growth and metabolic activity. As a result, the extent of lactic acid production is greatly reduced.

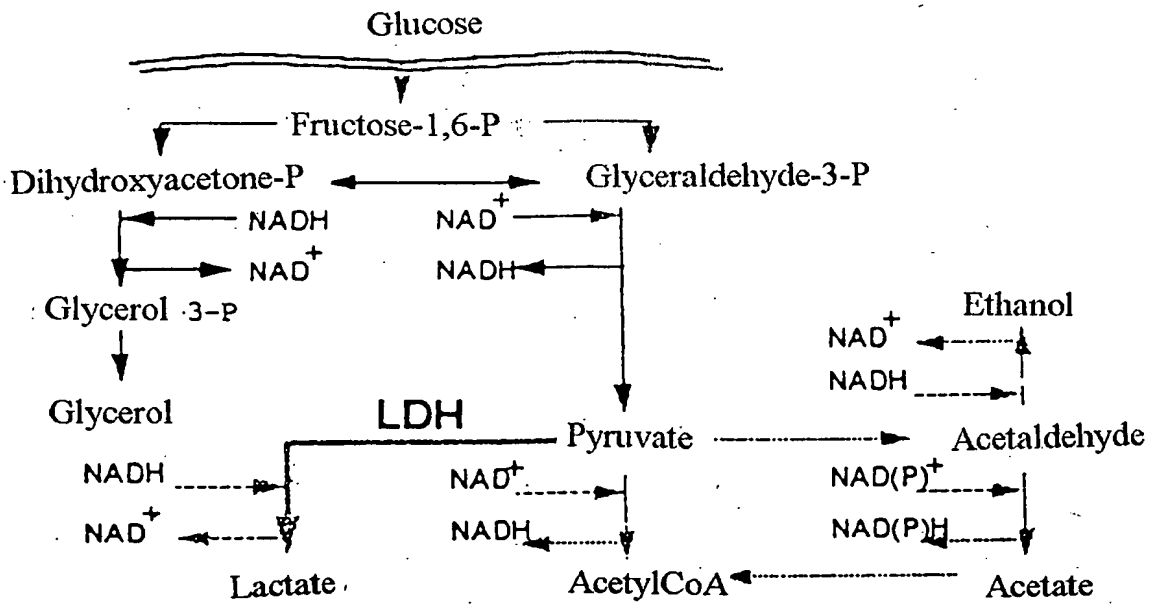
Therefore, the addition of $Ca(OH)_2$, $CaCO_3$, NaOH, or NH_4OH to neutralise the lactic acid and to thereby prevent the pH decrease is a conventional operation in industrial processes to

counteract the negative effects of free lactic acid accumulation.

These processes allow the production of lactate(s) by maintaining the pH at a constant value in the range of about 5 to 7; this is well above the pK_a of lactic acid, 3.86.

Major disadvantages are connected to the neutralisation of lactic acid during the fermentation. Mainly, additional operations are required to regenerate free lactic acid from its salt and to dispose of or recycle the neutralising cation; this is an expensive process. All the extra operations and expense could be eliminated if free lactic acid could be accumulated by microorganisms growing at low pH values, thus minimising the production of lactate(s).

It has been proposed the use of recombinant yeasts expressing the lactate dehydrogenase gene so as to shift the glycolytic flux towards the production of lactic acid.



FR-A-2 692 591 (Institut Nationale la Recherche Agronomique) discloses yeast strains, particularly Saccharomyces strains, containing at least one copy of a gene coding for a lactate

dehydrogenase from a lactic bacterium, said gene being under the control of sequences regulating its expression in yeasts.

Said strains may give both the alcoholic and the lactic fermentation and this so called "intermediate" or "balanced" fermentation could be exploited in areas such as brewing, enology, and baking.

Porro et al., (Biotechnol. Prog. 11, 294-298, 1995) have also reported the transformation of S. cerevisiae with a gene coding for bovine lactate dehydrogenase.

However, because of the high production of ethanol, the yield in the production of lactic acid for both the processes described was not considered to be competitive with that obtainable by the use of lactic bacteria.

Summary of the invention

According to a first embodiment, this invention provides yeast strains lacking ethanol production ability or having a reduced ethanol production ability and transformed with at least one copy of a gene coding for lactic dehydrogenase (LDH) functionally linked to promoter sequences allowing the expression of said gene in yeasts.

More particularly, this invention provides yeast strains lacking pyruvate decarboxylase (PDC) activity or having a reduced pyruvate decarboxylase activity and transformed with at least one copy of a gene coding for lactic dehydrogenase (LDH) functionally linked to promoter sequences allowing the expression of said gene in yeasts.

Other embodiments of the invention are the expression vectors comprising a DNA sequence coding for a lactic dehydrogenase functionally linked to a yeast promoter sequence. According to a further embodiment, the invention also provides a process for the preparation of lactic acid by culturing the yeast strains in a

fermentation medium containing a carbon source and recovering lactic acid from the fermentation medium.

Description of the invention

It has been found that very high yields in the production of lactic acid are obtained by engineered yeast strains so as to replace the ethanolic fermentation by lactic fermentation.

Such strains can be obtained by several methods, for instance by genetic engineering techniques aiming at inactivating or suppressing enzymatic activities involved in the production of ethanol, e.g. pyruvate decarboxylase and alcohol dehydrogenase activities.

Since pyruvate decarboxylase catalyses the first step in the alcohol pathway, yeast strains without or having a substantially reduced pyruvate decarboxylase activity and expressing a heterologous lactate dehydrogenase gene are preferred.

The expression of a LDH gene in yeast strains allows the production of lactic acid at acid pH values so that the free acid is directly obtained and the cumbersome conversion and recovery of lactate salts are minimized. In this invention, the pH of the fermentation medium may initially be higher than 4.5, but will decrease to a pH of 4.5 or less, preferably to a pH of 3 or less at the termination of the fermentation.

Any kind of yeast strain may be used according to the invention, but Kluyveromyces, Saccharomyces, species are preferred because these strains can grow and/or metabolize at very low pH, especially in the range of pH 4.5 or less; genetic engineering methods for these strains are well-developed; and these strains are widely accepted for use in food-related applications.

The term "reduced pyruvate decarboxylase activity" means either a decreased concentration of enzyme in the cell or reduced

or no specific catalytic activity of the enzyme.

According to the invention, it is preferred the use of strains wherein the pyruvate decarboxylase activity is or approaches zero but a reduced production for instance at least 60% lower, preferably at least 80% lower and even more preferably at least 90% lower than the normal of wild-type strains is acceptable.

Examples of Saccharomyces strains having a reduced PDC activity are available from ATCC under Acc.No. 200027 and 200028.

An example of Saccharomyces strain having no PDH activity has been described in Pronk JT. et al. (Microbiology. 140 (Pt 3):601-10, 1994).

An example of K. lactis having no PDC activity has been disclosed in Mol. Microbiol. 19 (1), 27-36, 1996.

The pyruvate decarboxylase activity can be measured by known methods, e.g. Ulbrich J., Methods in Enzymology, Vol. 18, p. 109-115, 1970, Academic Press, New York.

Suitable strains can be obtained by selecting mutations and/or engineering of wild-type or collection strains. The modulation of pyruvate decarboxylase activity by using nutrients supporting different glycolytic flow rates (Biotechnol. Prog. 11, 294-298, 1995) did not prove to be satisfactory.

A preferred method for decreasing or destroying the pyruvate decarboxylase activity and/or pyruvate dehydrogenase activity in a yeast strain according to the invention consists of the deletion of the corresponding gene or genes.

These deletions can be carried out by known methods, such as that disclosed in Bianchi et al., (Molecular Microbiol. 19 (1),27-36, 1996; Flikweert M.T. et al., Yeast, 12:247-257, 1996) by deletion or insertion by means of selectable markers, for instance the URA3

marker, preferably the URA3 marker from Saccharomyces cerevisiae. Alternatively, deletions, point-mutations and/or frame-shift mutations can be introduced into the functional promoters and genes required for the PDC activities. These techniques are disclosed, for instance, in Nature, 305, 391-397, 1983.

A Kluyveromyces lactis strain wherein the PDC gene has been replaced by the URA3 gene of S. cerevisiae has already been described in Molecular Microbiology 19(1), 27-36, 1996.

The gene coding for lactate dehydrogenase may be of any species and it may code for the L(+)-LDH or the D(-)-LDH. Alternatively, both types of LDH genes may be expressed simultaneously.

The transformation of the yeast strains can be carried out by means of either integrative or replicative vectors, linear or plasmidial.

The recombinant cells of the invention can be obtained by any method allowing a foreign DNA to be introduced into a cell, for instance transformation, electroporation, conjugation, fusion of protoplasts or any other technique known to the skilled in the art. Concerning transformation, various protocols have been described: in particular, it can be carried out by treating the whole cells in the presence of lithium acetate and of polyethylene glycol according to Ito H. et al. (J. Bacteriol., 153:163, 1983), or in the presence of ethylene glycol and dimethyl sulphoxyde according to Durrens P. et al. (Curr. Genet., 18:7, 1990). An alternative protocol has also been described in EP 361991. Electroporation can be carried out according to Becker D.M. and Guarente L. (Methods in Enzymology, 194:18, 1991).

The use of non-bacterial integrative vectors may be preferred when the yeast biomass is used, at the end of the fermentation process, as stock fodder or for other breeding, agricultural or

alimentary purposes.

In a particular embodiment of the invention, the recombinant DNA is part of an expression plasmid which can be of autonomous or integrative replication.

In particular, for both S. cerevisiae and K. lactis, autonomous replication vectors can be obtained by using autonomous replication sequences in the chosen host. Especially, in yeasts, they can be replication origins derived from plasmids (2μ, pKD1, etc.) or even chromosomal sequence (ARS).

The integrative vectors can be obtained by using homologous DNA sequences in certain regions of the host genome, allowing, by homologous recombination, integration of the vector.

Genetic tools for gene expression are very well developed for S. cerevisiae and described in Romanos, M.A. et al. Yeast, 8:423, 1992. Genetic tools have been also developed to allow the use of the yeasts Kluyveromyces species as host cells for production of recombinant proteins (Spencer Jf, et al., supra; Reiser J. et al., Advances in Biochemical Engineering-Biotechnology. 43, 75-102, 1990). Some examples of vectors autonomously replicating in K. lactis are reported, either based on the linear plasmid pKG1 of K. lactis (de Lovencourt L. et al. J. Bacteriol., 154:737, 1982), or containing a chromosomal sequence of K. lactis itself (KARS), conferring to the vector the ability of self replication and correct segregation (Das S., Hollenberg C.P., Curr. Genet., 6:123, 1982). Moreover, the recognition of a 2μ-like plasmid native to K. drosophilarum (plasmid pKD1- US 5 166 070) has allowed a very efficient host/vector system for the production of recombinant proteins to be established (EP-A- 361 991). Recombinant pKD1-based vectors contain the entire original sequence, fused to appropriate yeast and bacterial markers. Alternatively, it is possible to combine part of pKD1, with common S. cerevisiae

expression vectors (Romanos M.A. et al. Yeast, 8:423, 1992)(Chen et al., Curr. Genet. 16: 95, 1989).

Any yeast promoter, either inducible or constitutive, may be used according to the invention. To date, promoters used for the expression of proteins in S. cerevisiae are well described by Romanos et al. (supra). Promoters commonly used in foreign protein expression in K. lactis are S. cerevisiae PGK and PHO5 (Romanos et al., supra), or homologous promoters, such as LAC4 (van den Berg J. A. et al., BioTechnology, 8:135, 1990) and KlPDC (US 5 631 143). The promoter of pyruvate decarboxylase gene of K. lactis (KlPDC) is particularly preferred.

Vectors for the expression of heterologous genes which are particularly efficient for the transformation of Kluyveromyces lactis strains are disclosed in US 5 166 070, which is herein incorporated by reference.

Any yeast promoter, either inducible or constitutive, may be used according to the invention. Pyruvate decarboxylase gene promoters, preferably from Kluyveromyces species and even more preferably from Kluyveromyces lactis, disclosed in Molecular Microbiol. 19(1), 27-36, 1996, are particularly preferred.

For the production of lactic acid, the yeast strains of the invention are cultured in a medium containing a carbon source and other essential nutrients, and the lactic acid is recovered at a pH of 7 or less, preferably at a pH of 4.5 or less, and even more preferably at a pH of 3 or less. Since the pH of the culture medium is reduced, a lower amount of neutralizing agent is necessary. The formation of lactate salt is correspondingly reduced and proportionally less regeneration of free acid is required in order to recovery lactic acid. The recovery process may employ any of the known methods. Typically, the microorganisms are removed by filtration or centrifugation prior to lactic acid recovery. Known

methods for lactic acid recovery include, but are limited to, the extraction of lactic acid into an immiscible solvent phase or the distillation of lactic acid or an ester thereof.

The present invention offers the following advantages in the production of lactic acid:

1. When the fermentation is carried out at pH 4.5 or less, there is less danger of contamination by foreign microorganisms, as compared with the conventional process. Further, the fermentation facility can be simplified and the fermentation control can be facilitated.
2. Since less neutralizing agent is added to the culture medium for neutralization, there is correspondingly less need to use mineral acids or other regenerating agents for conversion of the lactate salt to free lactic acid. Therefore, the production cost can be reduced.
3. Since less neutralizing agent is added to the culture medium, the viscosity of the culture broth is reduced. Consequently, the broth is easier to process.
4. The cells separated in accordance with the present invention can be utilized again as seed microorganisms for a fresh lactic acid fermentation.
5. The cells can be continuously separated and recovered during the lactic acid fermentation, in accordance with the present invention, and hence, the fermentation can be carried out continuously.
6. Since the recombinant yeast strains lack ethanol production ability or have a reduced ethanol production, the production of lactic acid can be carried out with higher yield in comparison to yeast strains having a wild-type ability to produce ethanol.

Brief description of the Figures

Fig. 1. Diagram of the plasmid pVC1.

Fig. 2A., 2B. Diagram of the plasmid pK5MD8/7 and pKSEXH/16, respectively.

Fig. 3. Diagram of the plasmid pEPL2.

Fig. 4A. L(+)-Lactic acid production by PM6-7a[pEPL2] during growth on Glu-YNB based media. The residual glucose concentration at T=49 was not detectable. Production of D(-)-lactic acid was not detectable either. The LDH specific activity was higher than 3 U/mg of total cell protein along all the experiment.

(Δ) cells/ml; (-) pH value; (O) Ethanol production, g/l

( ) L(+)-Lactic acid production, g/l

Fig. 4B. L(+)-Lactic acid production from PM6-7a[pEPL2] during growth on YNB-based media. Medium was buffered at time T=0 (pH=5.6) using 200mM phosphate buffer. In this text batch, the pH value decreases much later than during the text batch shown in figure 4A. Neither the residual glucose concentration at T=49, nor the production of D-lactic acid were detectable. The LDH specific activity was higher than 3 U/mg of total cell protein along all the experiment.

(Δ) cells/ml; (-) pH value; (O) Ethanol production, g/l

( ) L(+)-Lactic acid production, g/l

Fig. 5A. L(+)-Lactic acid production from PM1/C1[pEPL2] during growth on Glu-YNB based media. The residual glucose concentration at T=60 was 12,01 g/l. Longer incubation times did not yield higher productions of both biomass and L(+)-Lactic acid. The LDH specific activity was higher than 3 U/mg of total cell protein along all the experiment.

(Δ) cells/ml; (-) pH value; (O) Ethanol production, g/l

( ) L(+)-Lactic acid production, g/l

Fig.5B. L(+)-Lactic acid production from the transformed PM1/C1[pEPL2] during growth on Glu-YNB based media. In this text batch, the pH value decreases much later than during the text

batch shown in figure 5A. The residual glucose concentration at T=87 was not detectable. The LDH specific activity was higher than 3 U/mg of total cell protein along all the experiment.

(Δ) cells/ml; (-) pH value; (O) Ethanol production, g/l

( ) L(+)-Lactic acid production, g/l

Detailed description of the invention

Definitions

Deletion: the procedures aiming at removing a gene or at least a part of same from a genome. Deletion is a mutagenesis technique (i.e. an instrument which is able to modify or cancel an enzymatic function in the cell), by means of frame-shift mutation (i.e. the alteration of a gene code) and point mutations (i.e. the alteration of a single point in the DNA sequence).

Expression vector: a DNA sequence containing a gene which is functionally linked to (i.e. under control of) a promoter able to control the gene expression in the host of interest.

LDH: lactate dehydrogenase: an enzyme which is able to transform pyruvate and NADH in lactic acid and NAD+. L(+)-LDH provides L(+)-lactic acid, whereas D(-)-LDH provides D(-)-lactic acid.

PDC: pyruvate decarboxylase, an enzyme which is able to transform pyruvate in acetaldehyde, which is the first step to produce ethanol in yeast.

Promoter: a DNA sequence able to control the expression of a gene fused downstream. Such an expression can be continuous (constitutive promoter) or controlled by determined signals (inducible promoter).

Repair minus; a particular strain of Escherichia coli, which is not able to carry out all repairing works of its genome, which is suitable for operations of recombinant DNA.

Selectable Marker: a gene which allows to discriminate the

cells with or without any particular plasmid transporting it,

Vector: an extra-genomic DNA sequence which is kept inside the host during the cell doubling (segregation). The vectors are inserted inside the host (transformation) and are capable of autonomously replicating (replicative vector, plasmid) and are integrated in the genome of the host (integrative vector), which becomes part of the very genome.

Site-directed mutagenesis of the bovine lactate dehydrogenase gene (LDH-A)

In order to isolate from the full length cDNA the coding sequence of the bovine enzyme LDH-A (EC 1.1.1.27), a classical site directed mutagenesis, (J. Biol. Chem. 253:6551, 1978, Meth. Enzymol. 154:329, 1987), was performed. Oligonucleotides-driven site-specific mutagenesis is based on the *in vitro* hybridization of a single-stranded DNA fragment with a synthetic oligonucleotide, which is complementary to the DNA fragment except for a central mismatching region in correspondence of the nucleotide or nucleotides that are to be substituted.

In order to introduce by mutagenesis a Xba I restriction enzyme site at position 67, a 1743 bp bovine LDH cDNA was excised from the plasmid pLDH12 (Ishiguro et al., Gene, 91 281-285, 1991) by digestion with Eco RI and Hind III restriction enzymes (New England Biolabs) and was then cloned in the pALTER-1 (Promega, cat # 96210; lot # 48645, 1996) expression vector, which contains M13 and R408 bacteriophages origin of replication and two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is functional. The other (i.e., ampicillin resistance) has been inactivated. After infecting a culture of E. Coli with R408, previously transformed with the above described construct, phage particles containing single-stranded DNA (ssDNA) plasmid were obtained. Mutagenesis is based on the use of two oligonucleotides

(Table 1). The one is able to restore ampicillin (oligoAMP; kit Promega); the other is suitably planned with one or more mismatches which are necessary to determine the desired substitution of aminoacids in the gene product of interest (oligoLDH). After synthesizing in vitro the second DNA strand, a culture of the strain E. Coli is transformed by selection of a L+B ampicillin liquid medium (Sambrook et al., Molecular Cloning: A Laboratory Manual edited by Sambrook et al., Cold Spring Harbor Laboratory Press). Said strain is mutated in the DNA repairing apparatus (BMH 71-18 mutS; kit Promega), in order to keep in vivo the mismatches inserted along with the synthetic oligonucleotides. A second round of transformation in JM109 (kit Promega) ensures a correct segregation of mutant and wild-type plasmids, giving high proportions of plasmids with respect to the mutated construct. Further details can be found in the kit datasheet.

The plasmid obtained, containing the mutated cDNA for the bovine LDH, was denominated pVC1 (Fig. 2).

A person skilled in the art will be aware that any other method selected to carry out a mutagenesis reaction can be employed within the limits of the present invention.

OLIGONUCLEOTIDES	SEQUENCE
OligoAMP	5'-GTTGCCATTGCTGCAGGCATCGTGGTG-3'
OligoLDH	5'-CCTTTAGGGTCTAGATCCAAGATGGCAAC-3'

Table 1: Nucleotide sequence of the synthetic oligonucleotides used for the site-directed mutagenesis. The underlined sequence in the oligoLDH show the Xba I restriction site introduced by mutagenesis.

Construction of the pEPL2 replicative vector containing the KIPDCA promoter and the bovine LDH cDNA.

The KIPDCA promoter and the coding sequence were subcloned as a 4 Kbp HindIII fragment from a K. lactis genomic library clone complementing the rag 6 mutation of K. lactis (Bianchi et al., Mol. Microbiol., 19: 27-36, 1996). The promoter region was subcloned into Sal I and Xba I sites of the vector pBleuscript II KS (Stratagene, LaJolla, Ca # 212205) with T4 DNA ligase using molecular cloning standard procedure (Sambrook et al., Molecular Cloning, supra). The cDNA of the bovine LDH sequence, isolated as a Xba I-Hind III fragment of 1675 bp from the pVC1 vector, was cloned by the same method in the corresponding cloning sites of the vector pBleuscript II KS. GM82. The two new vectors, called respectively pKSMD8/7 and pKSEXH/16 (Fig 2A and 2B)were transformed into *E. coli* strain (*dam*$^-$ *dcm*$^-$).

KIPDCA promoter and cDNA of bovine LDH sequence, isolated as Sal I-Xba I fragments, respectively from pKSMD8/7 and pKSEXH/16, were isolated according to the methods described above and ligated in vitro with T4 DNA ligase at room temperature in the presence of Sal I endonuclease in order to allow the ligation at Xba I ends. The ligation product was cloned in Sal I cloning site of pE1 vector (Bianchi M.et al., Curr. Genet. 12: 185-192, 1987; Chen X. J. et al., Curr. Genet. 16: 95-98, 1989 and US # 5166070). This plasmid is based on the YIp5 integrative plasmid containing the Saccharomyces cerevisiae genetic marker URA3 and on the pKD1 plasmid (US # 5166070). This plasmid was isolated from Kluyveromyces drosophilarum and has a functional organization similar to the S. cerevisiae 2μ DNA and is able to replicate in a stable way in Kluyveromyces lactis cells (Chen X. J. et al., supra). The URA3 marker on the plasmid allows the complementation of the K. lactis uraA1-1 mutation (de Louvencourt et al., J. Bacteriol. 154: 737-742 (1982)).

The vector obtained was called pEPL2 (Fig. 4) and used to transform *E. coli* DH5-alfa strain (Life Technologies Inc., Gaitherburg, MA).

Isolation of the K. lactis PMI/C1 strain

Deletion of the KIPDCA gene in the PM6-7A yeast strain (MAT a, adeT-600, uraA1-1)(Wesolowski et al., Yeast 1992, 8: 711) yielded the strain PMI. Stable ura+ mutants grow on glucose-containing media; PDC activity and ethanol production are not detectable (Bianchi M.M., et al.,(1996), supra).

$1x10^7$-$3x10^7$ cells from a stationary culture of PMI yeast cells were plated on synthetic medium containing 5-fluoroorotic acid (McCuster and Davis, Yeast 7: 697-608, 1991). After 5 days incubation at 28° C some ura- mutants were isolated. One of these mutants obtained, called PMI/C1, resulted mutated in the URA3 gene previously introduced by integrative transformation, as it resulted both from a complementation test by transformation with an URA3 gene-containing plasmid (Kep6 vector; Chen et al.., J. Basic Microbiol. 28: 211-220 (1988)) and from the formation of ura- diploids by crossing Mata, uraA1-1 strains (such as MW98-8C: Matα, uraA1-1, arg,lys, rag1,rag2). The genotype of PMI/C1 is the following: MATa, adeT-600, uraA1-1, pdcA::ura3.

Transformation of Kluyveromyces yeast PM6-7A and PMI/C1 with the vectors pEPL2 and pEPL4.

PM6-7A and PMI/C1 cells, grown in YPD medium (1% w/v yeast extract; 2% w/v peptone; 2% w/v glucose) until a concentration of $0.5x10^8$ cells/ml was obtained, was harvested, washed once in water, twice in 1M sorbitol, and resuspended in 1M sorbitol at a concentration of $2x10^9$ cells/ml. Cells were electroporated (7.5 KV/cm, 25 μF, 200 Ω: GenePulser, Biorad) in the presence of 5-10 microg of pEPL2 or pEPL4. Selection of URA^+ transformants was carried out in synthetic solid medium

without uracil (0.7 % w/v Yeast Nitrogen Base, 2 w/v % glucose, 200 mg/l adenine, 2 w/v % agar).

Batch culture of PM6-7A[pEPL2] and PMI/C1[pEPL2] transformants with pEPL2 plasmid

Some isolated clones were tested in batch conditions in minimum synthetic medium (1.3% w/v Yeast nitrogen base -aa (Difco), 200 mg/l adenine, 50 g/l glucose) either buffered or not at pH 5,6 with 200 mM phosphate buffer.

The cells were preinoculated in the same medium of the experiment and, once they started growing actively, they were inoculated in Erlenmeyer flasks (vol. 300 ml), containing 100 ml of fresh medium.

The Erlenmeyer flasks were incubated in Dubnoff bathtubs at 30° C, 150 rpm, monitoring the fermentation at regular intervals.

Cellular growth was calculated with an electronic counter for particles (Coulter Counter ZBI, Porro et al., Res. Microbiol. (1991), 142, 535-539), after removing any cellular aggregating agents, by sonicating the suspension for 10 seconds (Sonicator Fisher 300, medium push rod, 35% Power.

LDH activity dosage

About 10^8 cells were harvested, washed in 50mM phosphate buffer, pH 7.5, and resuspended in the same buffer. Cells were lysed with 5 cycles of vigorous vortexing in the presence of glass microbeads (diameter 400 µm, SIGMA, G-8772) at 4° C. Cellular debris were removed by centrifugation (Eppendorf, Hamburg, D 5415 C, 13600 RCF, 10 min), and protein extracts' concentration was determined by Micro Assay, Biorad, Hercules, Ca (cat. 500-0006).

About 0.2 mg of extract were tested for LDH activity using SIGMA kit DG1340-UV, according to manufacturer's instructions.

Metabolites dosage in the growth medium.

Samples from the growth medium, obtained after removing cells by centrifugation, were analysed for the presence of glucose, ethanol, L- and D-lactic acid using kits from Boehringer Mannheim, Mannheim DE, (#. 716251, 176290, and 1112821 respectively), according to manufacturer's instructions.

Experimental batch tests are shown in figures 4A, 4B and figures 5A, 5B. The results obtained are summarized in Table 2.

The yield is the amount of lactic acid produced (gr/l) divided by the amount of glucose consumed (gr/l). The percentage of free lactic acid is obtained from the Henderson-Hasselbalch equation:

$pH = pK_a + \log[(\%Lactate)/(\%Free\ Lactic\ Acid)]$,

where the pK_a for lactic acid is 3.86.

TABLE 2: Lactic acid production by PM6-7A[pEPL2] and PMI/C1[pEPL2]

	phosphate buffer	lactic acid (g/l)	Yield (g/g)	Final PH	Free lactic acid
PM6-7A[pEPL2]	-	1.2	0.020.	2.0	99
PM6-7A[pEPL2]	+	4.3	0.087	3.0	88
PMI/C1[pEPL2]	-	2.0	0.052	2.3	97
PMI/C1[pEPL2]	+	11.4	0.233	2.9	90

Lactic Acid Production by PMI/C1[pEPL2] in a Stirred-tank Fermentor

Lactic acid production by PMI/C1[pEPL2] was further tested by cultivation in a 14-liter stirred-tank fermentor. Inocula for the fermentations were prepared by pre-culturing PMI/C1[pEPL2] in 50 mL of minimum synthetic medium (1.3% w/v Yeast Nitrogen Base -aa (Difco, Detroit, MI), 200 mg/L adenine, 5 g/L ammonium

sulfate, 50 g/L glucose) in a 250 mL baffled Erlenmeyer flasks for 30 hr at 30° C and 300 rpm in an incubator shaker (Model G-24, New Brunswick Scientific Co., Inc., Edison, NJ). 80 ml of the total inocula were transferred to a 14-liter stirred tank fermentor (BioFlo 3000 System, New Brunswick Scientific Co., Inc., Edison, NJ) containing 8 liters of nutrient medium (30 g dry solids/L light corn steep water, A.E. Staley Manufacturing Co., Decatur, IL; 10 g/L Difco yeast extract, Difco, Detroit, MI; 200 mg/L adenine, 50 g/L glucose). The fermentor was kept at 30° C, agitated at 400 rpm, and aerated at 2 liters/min throughout. Antifoam (Antifoam 1520, Dow Corning Corp., Midland, MI) was added as needed to control foaming. Glucose was fed as needed to maintain a residual concentration in the fermentation medium of about 25-50 g/L. When controlled, the pH was maintained by automatic addition of 14.8 M ammonium hydroxide in water. Lactic acid production at acidic pH was tested as follows: (1) The fermentation pH was controlled at 4.5 throughout the fermentation. (2) The initial fermentation pH was controlled at 4.0 until 80 mL of 14.8 M ammonium hydroxide were added. Then pH control was discontinued. (3) The initial fermentation pH was 5.0 and no neutralizing agent was added during the fermentation. The results are shown in Table 3. The elapsed time was measured from the time of inoculation. Samples from the fermentation, obtained after removing cells by filtration, were analyzed for the presence of glucose and L(+)-lactic acid using a YSI Model 2700 Select Biochemistry Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). Ethanol, measured by gas chromatography, was not detected in any of the fermentations. Yield and % free lactic acid were calculated as previously described.

Table 3. Lactic Acid Production by PMI/C1[pEpI2] in a

Bioreactor.

	Elapsed Time (hr)		NH_4OH Added (M)	Lactic Acid (g/L)	Yield (g/g)	Final pH	% Free Lactic Acid
Case 1	137	1.31		109	0.59	4.5	19
Case 2	97	0.14		35	0.44	3.0	88
Case 3	72	0		29	0.35	2.8	92

CLAIMS

1. A yeast strain lacking ethanol production ability or having a reduced ethanol production ability transformed with at least one copy of a gene coding for lactic dehydrogenase, functionally linked to promoter sequences allowing the expression of said gene in yeasts.

2. A yeast strain according to claim 1 lacking pyruvate decarboxylase or having a substantially reduced pyruvate decarboxylase activity.

3. A yeast strain according to claims 1 or 2 selected from the species Saccharomyces or Kluyveromyces.

4. A yeast strain according to claim 3 belonging to the species Kluyveromyces.

5. A yeast strain according to claim 4, which is a Kluyveromyces lactis strain.

6. A yeast strain according to anyone of claims 1 to 5, transformed with one or more genes coding the enzyme lactate dehydrogenase.

7. A yeast strain according to claim 6 transformed with a gene coding for the bovine lactate dehydrogenase.

8. A yeast strain according to any one of claims 1 to 7, wherein the gene coding for the lactate dehydrogenase is integrated in the yeast genome.

9. A yeast strain according to any one of claims 1-8 which has been transformed by an expression vector comprising a promoter sequence and a DNA sequence coding for lactate dehydrogenase under the regulation of said promoter sequence

10. A yeast strain according to claim 9 wherein the promoter sequence is a pyruvate decarboxylase gene promoter.

11. A yeast strain according to claim 10 wherein the promoter

sequence is a Kluyveromyces pyruvate decarboxylase gene promoter.

12. A yeast strain according to any one of claims 1-11 wherein the gene or the genes coding for pyruvate decarboxylase has or have been disrupted by deletion or insertion by means of selectable marker(s).

13. A yeast strain according to claim 12, wherein the selectable marker is a URA3 marker.

14. A yeast strain according to claim 13 wherein the selectable marker is the URA3 marker from Saccharomyces cerevisiae

15. An expression vector which comprises a DNA sequence coding for a lactic dehydrogenase functionally linked to a yeast promoter sequence.

16. A vector according to claim 15, in which the promoter sequence is a promoter of the Kluyveromyces pyruvate decarboxylase gene.

17. A vector according to claim 16, in which the promoter sequence is a promoter of the Kluyveromyces lactis pyruvate decarboxylase gene.

18. A process for the production of lactic acid which consists of the fermentation of the strains of claims 1-14 in a fermentation medium containing a carbon source and the recovery of the lactic acid from the fermentation medium.

19. A process according to claim 18, in which the fermentation medium has a pH value equal to or lower than 7.

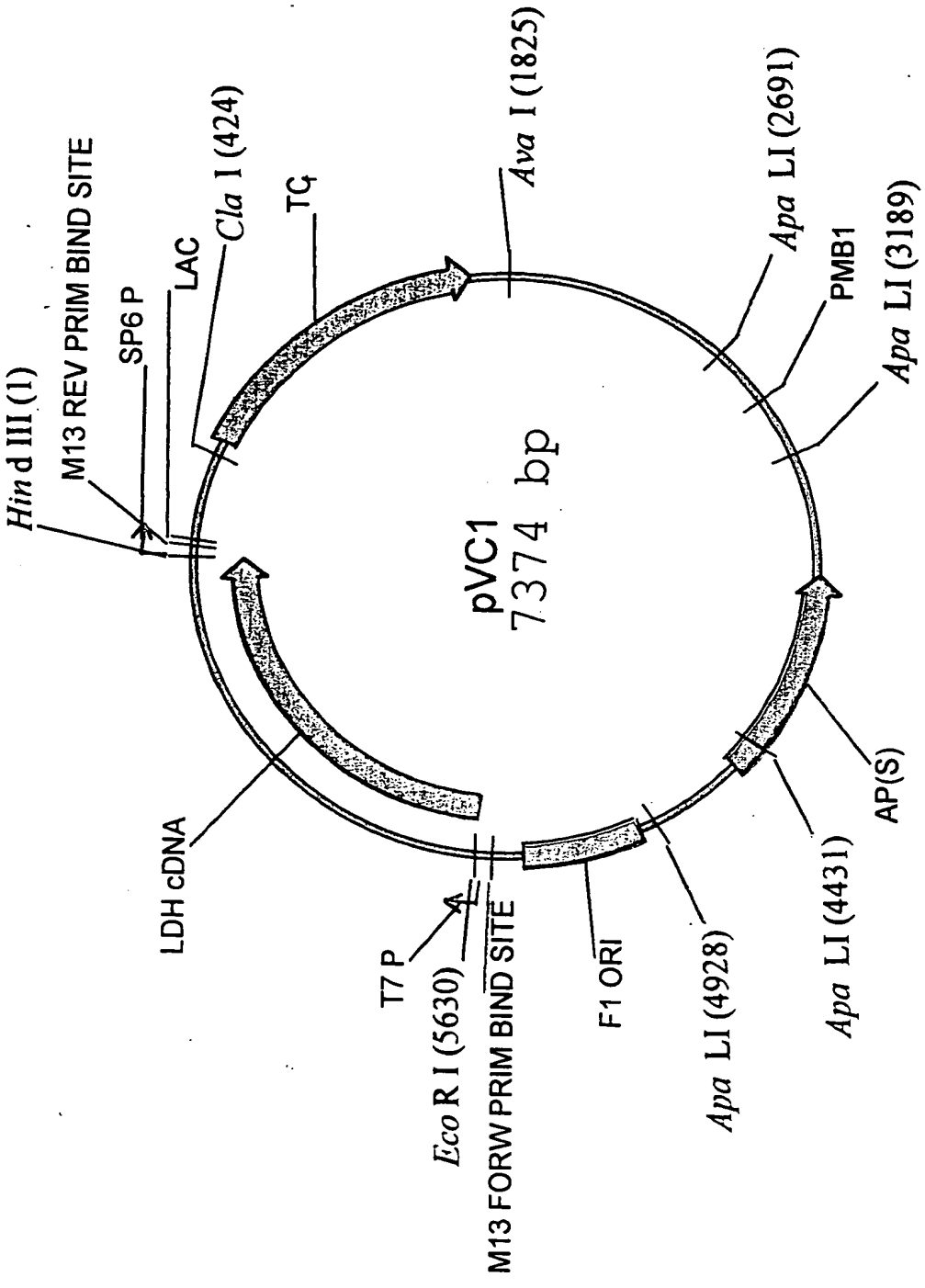
20. A process according to claim 19, in which the pH value is equal to or lower than 4.5.

21. A process according to claim 20, in which the pH value is equal to or lower than 3.

Milan, 12 September 1997

The Agent
(Marco Spadaro)
BIANCHETTI BRACCO MINOJA S.r.l.
(signature)

Figure 1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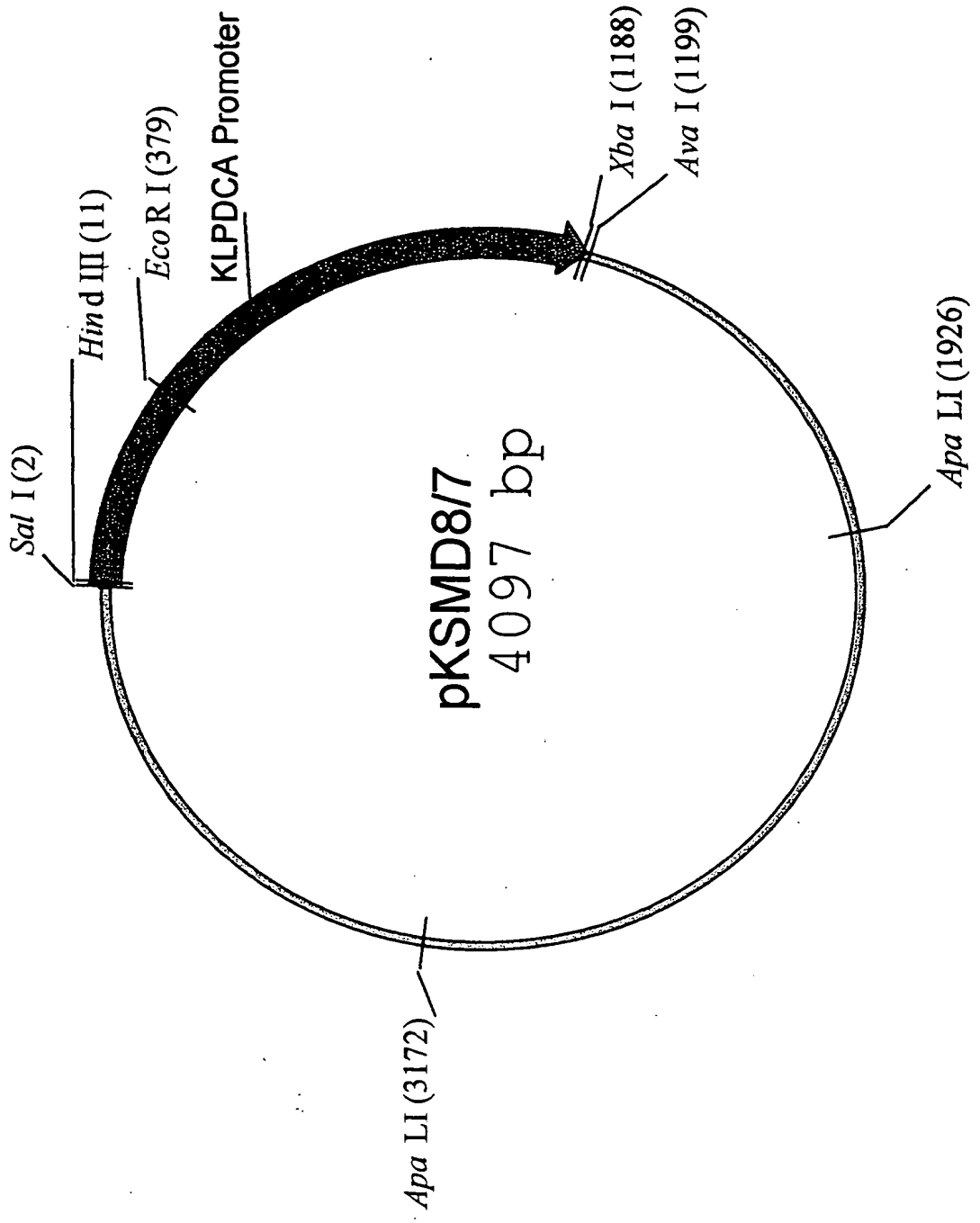
M13 REV PRIM BIND SITE
SP6 P
LAC
Cla I (424)
TG
Ava I (1825)
Apa LI (2691)
PMB1
Apa LI (3189)
AP(S)
Apa LI (4431)
Apa LI (4928)
F1 ORI
M13 FORW PRIM BIND SITE
Eco R I (5630)
T7 P
LDH cDNA

Figure 2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4097 bp
KLPDCA Promoter
Sal I (2)
Hin d III (11)
Eco R I (379)
Xba I (1188)
Ava I (1199)
Apa LI (1926)
Apa LI (3172)

Figure 2A

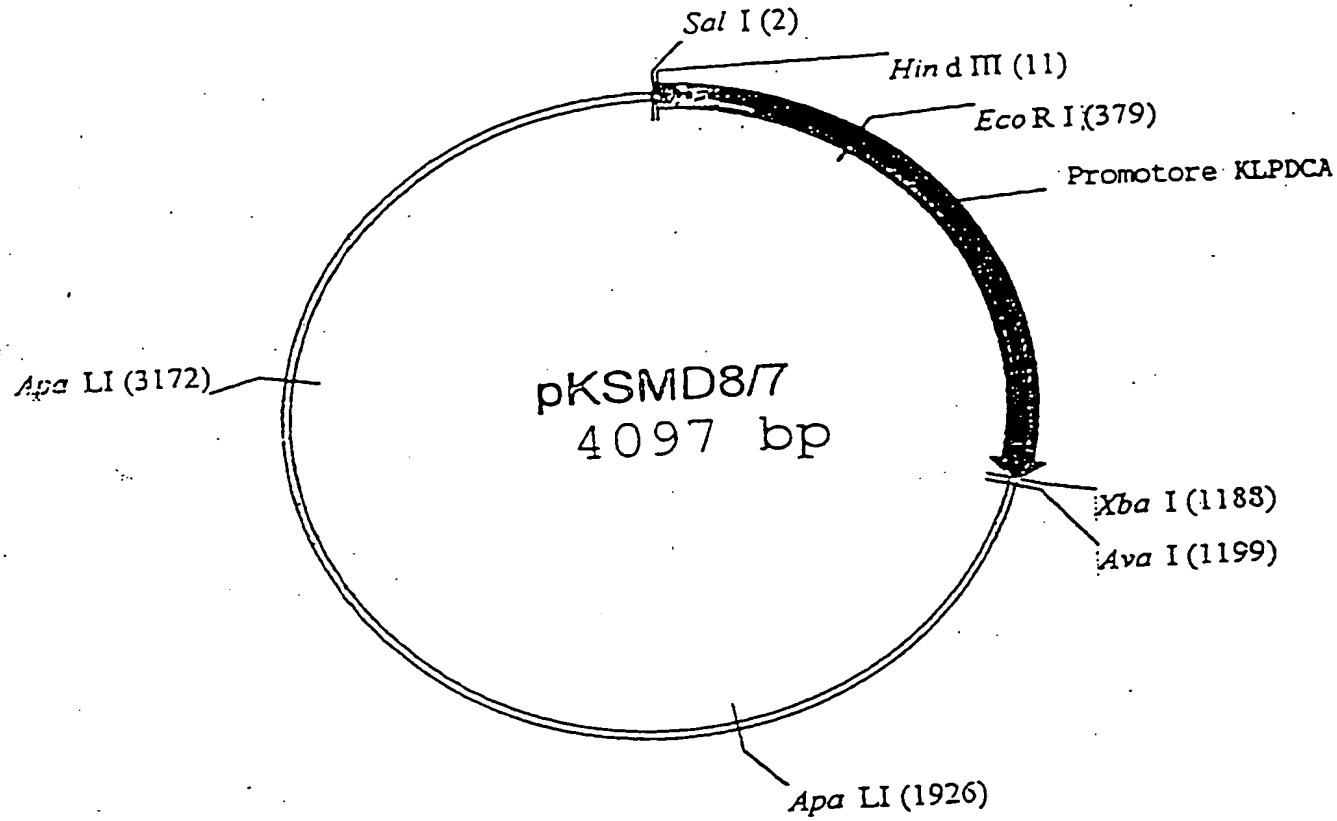
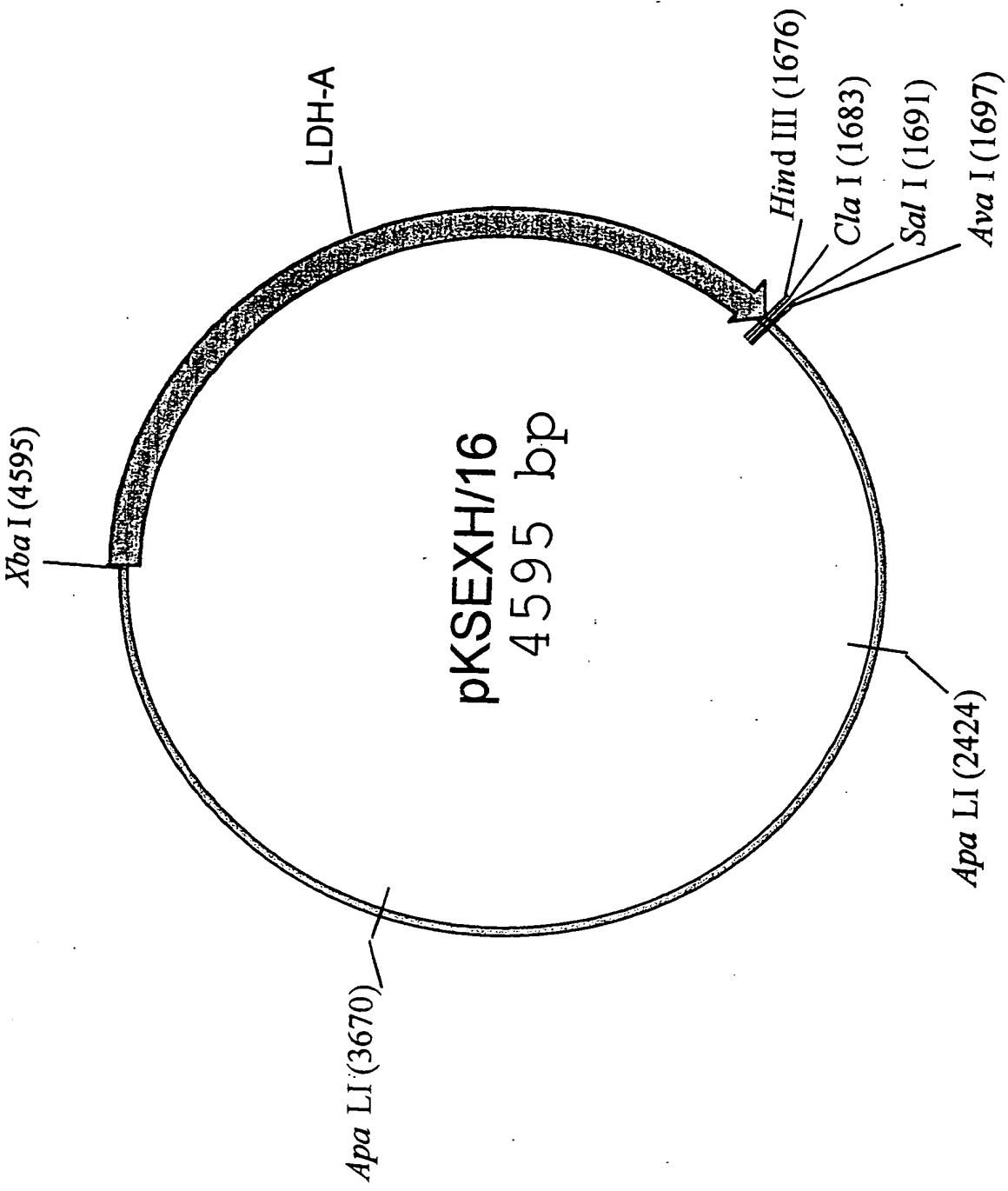

Sal I (2)
Hin d III (11)
Eco R I (379)
Promotore KLPDCA
Apa LI (3172)
pKSMD8/7
4097 bp
Xba I (1188)
Ava I (1199)
Apa LI (1926)

Figure 2B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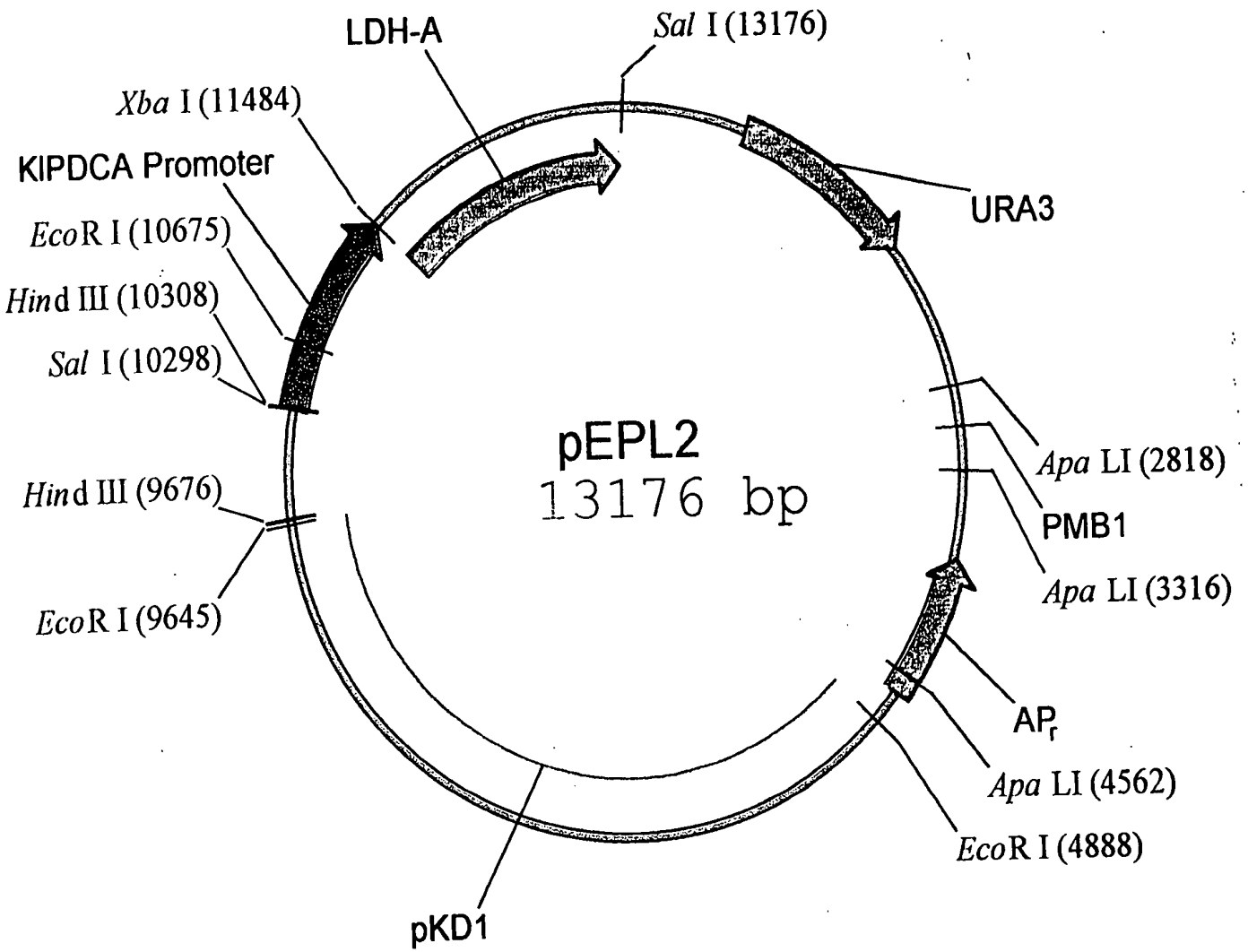
Cla I (1683)
Sal I (1691)
Ava I (1697)
Xba I (4595)
pKSEXH/16
4595 bp
Apa LI (2424)
Apa LI (3670)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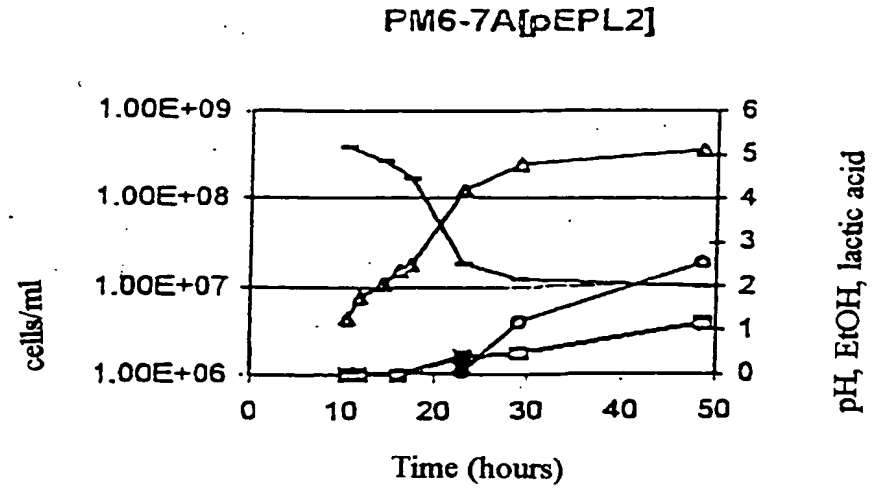
Sal I (13176)
Xba I (11484)
KIPDCA Promoter
EcoR I (10675)
Hind III (10308)
Sal I (10298)
URA3
pEPL2
13176 bp
Hind III (9676)
EcoR I (9645)
Apa LI (2818)
PMB1
Apa LI (3316)
AP$_r$
Apa LI (4562)
EcoR I (4888)
pKD1

Figure 3

Figure 4A & 4B


PM6-7A[pEPL2]
1.00E+09
1.00E+08
1.00E+07
1.00E+06
cells/ml
0
10
20
30
40
50
Time (hours)
6
5
4
3
2
1
0
pH, EtOH, lactic acid

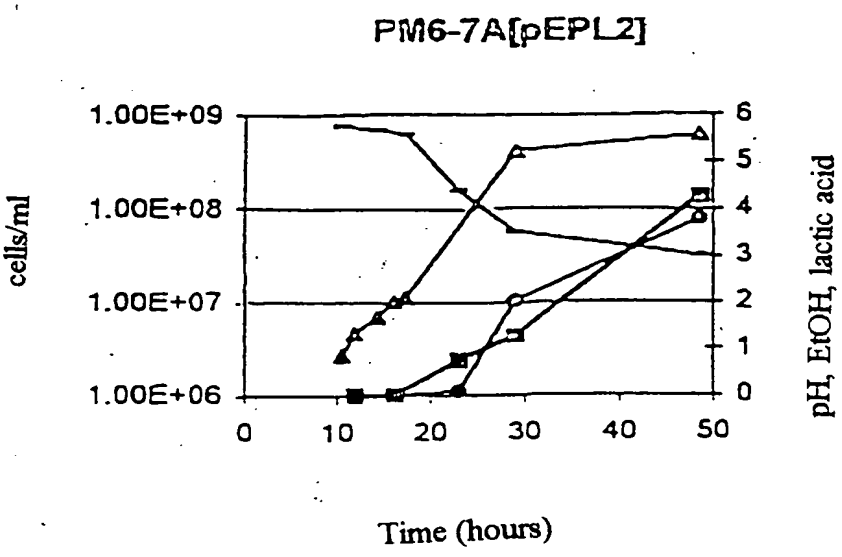
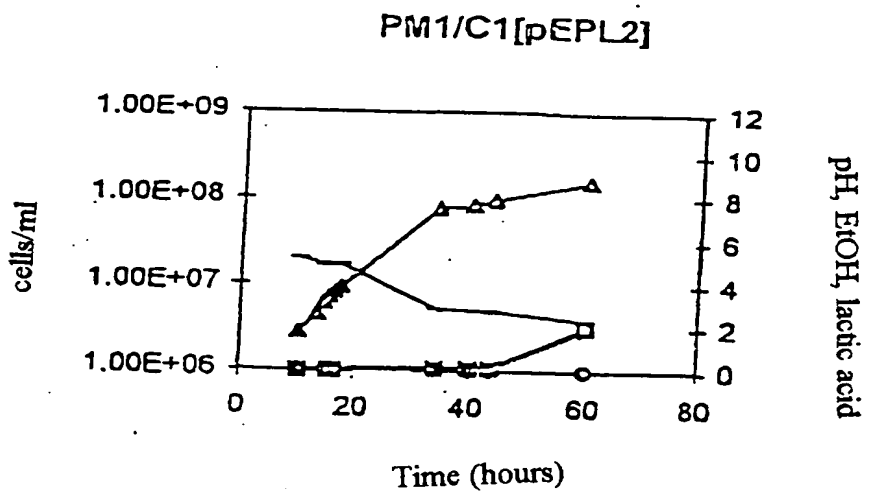
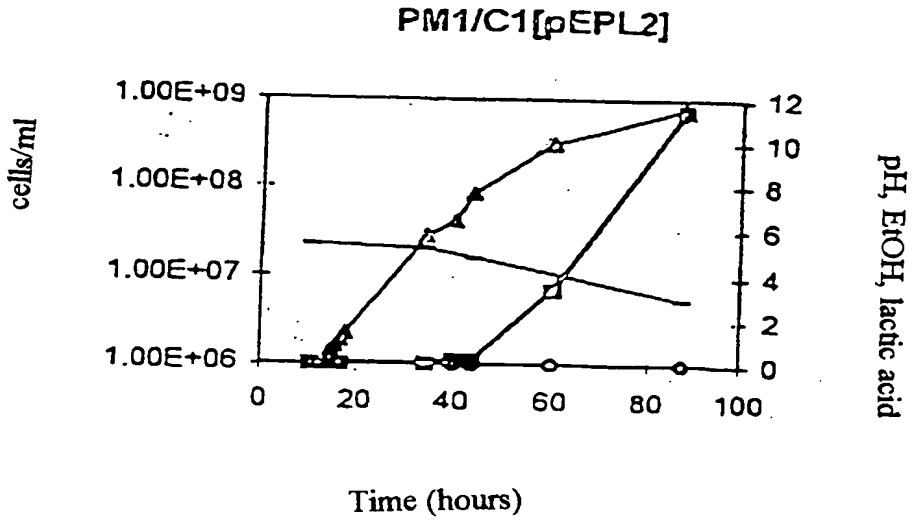

PM6-7A[pEPL2]
1.00E+09
1.00E+08
1.00E+07
1.00E+06
cells/ml
0
10
20
30
40
50
Time (hours)
6
5
4
3
2
1
0
pH, EtOH, lactic acid

Figure 5A & 5B


PM1/C1[pEPL2]
1.00E+09
1.00E+08
1.00E+07
1.00E+06
cells/ml
0
20
40
60
80
12
10
8
6
4
2
0
pH, EtOH, lactic acid
Time (hours)


PM1/C1[pEPL2]
1.00E+09
1.00E+08
1.00E+07
1.00E+06
cells/ml
0
20
40
60
80
100
12
10
8
6
4
2
0
pH, EtOH, lactic acid
Time (hours)

I, **Cecilia BIANCHETTI,** of Via Goldoni 1 – 20129 Milano, Italy, do hereby declare that I well understand the Italian and the English languages, and that the foregoing is a full, true and faithful translation made by me of the Italian patent application **MI97A 002080** filed on September 12, 1997

Milan, May 23, 2001

SIGNATURE: Cecilia Bianchetti